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Platelet von Willebrand Factor in Hermansky-Pudlak Syndrome

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The Hermansky-Pudlak Syndrome (HPS) is an autosomal recessive inherited disorder characterized by oculocutaneous albinism, tissue accumulation of ceroid pigment, and a mild to moderate bleeding diathesis attributed to storage-pool deficient (SPD) platlets. Patients have platelet aggregation and release abnormalities. In addition, low levels of plasma von Willebrand factor (vWF) antigen in some HPS patients have been associated with a greater bleeding tendency than would be predicted from either condition alone. Other HPS patients have severe bleeding despite normal levels of plasma vWF, suggesting that at least one additional factor is responsible for their bleeding diathesis. Because platelet vWF levels have been well correlated with clinical bleeding times in patients with von Willebrand's disease, we have measured the platelet vWF activity and antigen levels in 30 HPS patients and have attempted to correlate their clinical bleeding with these values. The platelet vWF activity levels in patients was significantly lower than that of normal subjects ($P < 0.0001$). The patients as a group also had slightly lower values of plasma vWF activity when compared with normals ($P \sim 0.03$). In 11 of the HPS patients, the multimeric structure of plasma vWF showed a decrease in the high molecular weight multimers and an increase in the low molecular weight multimers. In correlating the platelet and plasma vWF values with the bleeding histories, we were not able to show a predictable relationship in the majority of the patients. Am. J. Hematol. 59:115–120, 1998. © 1998 Wiley-Liss, Inc.

Key words: Hermansky-Pudlak Syndrome; von Willebrand factor; bleeding diathesis

INTRODUCTION

In 1959 Hermansky and Pudlak [1] reported two unrelated patients exhibiting oculocutaneous albinism and a moderately severe hemorrhagic disorder. Both patients had congenital nystagmus, large reticular cells with an unusual pigment in the bone marrow, and prolonged bleeding times. In their original studies these investigators were not able to exclude the possibility that both vascular and platelet defects contributed to the pathogenesis of bleeding in these patients. Subsequent studies demonstrated that these and similar patients hava a platelet storage pool deficiency (SPD), a qualitative and quantitative defect affecting the platelet dense granules [2,3]. Their platelets characteristically fail to aggregate with collagen and do not exhibit a second wave of platelet aggregation with epinephrine and adenosine 5′-diphosphate (ADP) [3–7]. Additionally, although the number of platelet α-granules is normal in patients with Hermansky-Pudlak syndrome (HPS) [2,3], α-granule release has been reported to be impaired in one patient [3,8]. The content of platelet lysosomal β-n-acetylglucosaminidase and β-glucuronidase is normal [2,3].

The gene for HPS has been mapped to chromosome 10q23 [9] in a Puerto Rican population and was recently cloned and sequenced [10]. Gahl et al. (submitted for publication) have observed that the 16 base-pair duplication in exon 15 of the HPS gene on 10q23 occurred in both alleles of 25 Puerto Rican patients and was absent from all the 22 non-Puerto Rican patients studied. The distribution of HPS is worldwide, although the highest prevalence occurs in Puerto Rico. Clinically, the oculo-

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cutaneous pigmentation of HPS patients ranges from an almost total absence of pigment to an amount that is nearly normal [9]. The variability in phenotype suggests that HPS exhibits locus heterogeneity or variable expressivity. The accumulation of ceroid/lipofuscin-like material in cells and tissues correlates with restrictive lung disease, granulomatous colitis, and renal failure, the most frequent serious complications in HPS [11]. Shallreuter et al. [12] have reported that, unlike the Puerto Rican HPS patients, Swiss HPS patients lack the ceroid material.

Some HPS patients bleed more than others with similar platelet abnormalities, and synergism between HPS and von Willebrand's disease has been suggested by Daniels et al. [13] and Witkop et al. [14]. These investigators have observed that the risk of bleeding in HPS patients increases when the level of plasma vWF antigen is ⩽70 U/dl [14]. Studies of platelet vWF in HPS have been limited. Martin and coworkers [15] reported normal amounts and normal multimeric composition of surface-associated platelet vWF in one HPS patient. Other investigators studying vWF released from platelets of two HPS patients could not detect vWF in electrophoretic studies for vWF multimers despite normal plasma vWF multimeric structure [16]. We have examined the platelet and plasma vWF ristocetin cofactor activity, antigen level, and multimeric structure in HPS patients in an attempt to define the role of vWF in the hemorrhagic tendency of patients with HPS.

MATERIALS AND METHODS

Patients

The patients in this study were diagnosed with HPS before admission of the Clinical Center at the National Institutes of Health. Patients or their parent or guardian gave informed consent to participate in this protocol, which was reviewed and accepted by the Institutional Review Board of the National Institute of Child Health and Human Development. The purpose of the protocol was to assess the medical complications of HPS and to determine the basic defect(s) in HPS. The possible risks and benefits of participation in this investigation were explained to each patient. The patients ranged in age from four to 54 years; 22 patients were male and 22 were female. Twenty-six of the patients studied were Puerto Rican and 18 were non-Puerto Rican.

Blood Collection

Blood was obtained from patients and normal donors through a 19- or 21-gauge needle using at two-syringe technique. All normal donors were adults (>18 years of age). For determination of plasma vWF activity, vWF antigen level, vWF multimers, and Factor VIII, whole blood was anticoagulated with sodium citrate (10.9 mM final concentration). Cell-free plasma was prepared by centrifugation at 2,000g for 10 min at 4°C. To obtain platelets for vWF activity, antigen, and multimer determinations, nine ml of whole blood were added to a polypropylene tube containing one ml of anticoagulant (final concentration 10.9 mM sodium citrate, one mM ethylenediaminetraacetic acid). To assess platelet vWF activity and antigen, platelets were separated from plasma components by centrifugation on a Larcoll® gradient (Sigma, St. Louis, MO) and platelet lysates were prepared as described previously [17]. Blood for platelet aggregation studies was anticoagulated with sodium citrate (final concentration 10.9 mM). Platelet rich plasma (PRP) was obtained by centrifugation at 750g for 3 min at 24°C. The PRP was removed, and the remaining blood was centrifuged at 2,000g for 10 min at 24°C. The platelet poor plasma (PPP) was used to adjust the platelet count of the PRP to 200,000/μl.

Plasma and Platelet vWF and Coagulation Assays

Plasma and platelet vWF activity (ristocetin cofactor) was determined by using normal formalinized platelets or lyophilyzed platelets (Bio-Data, Horsham, PA) in a tilt tube assay as described previously [18]. vWF antigen levels were quantitated by electroimmunodiffusion on agarose plates containing monospecific rabbit polyclonal antihuman vWF (Dako, Carpinteria, CA) [18] or by electroimmunodiffusion using Assera®-Plate reagents (Diagnostica Stago, Asnieres-sur-Seine, France). vWF multimers were visualized by agarose gel electrophoresis as described previously [17]. Densitometric analysis of vWF multimer autoradiographs was performed on a model GS 670 imaging densitometer equipped with Molecular Analyst™/Macintosh software (BioRad Laboratories, Hercules, CA). Factor VIII activity was measured by a one-stage method based on the activated partial thromboplastin time [18].

Bleeding Time

Bleeding times were performed using the automated Surgicutt® incision-making instrument (International Technidyne Corporation, Edison, NJ).

Platelet Aggregation and Release Reaction

Platelet aggregation studies were performed with a lumi-aggregometer (Chrono-log, Havertown, PA). Platelets from normal donors were used as controls. Four tenths of a milliliter of PRP adjusted to a platelet count of 200,000/μl was placed in a siliconized cuvette with a stir bar. After a baseline was established, 50 μl of luciferin-luciferase reagent (Sigma, St. Louis, MO) were added to detect dense granule adenosine 5′ triphosphate disodium (ATP) release. The following agonists were added: ADP (Sigma, St. Louis, MO), final concentrations 3.8 μM and 16.4 μM; epinephrine (Parke-Davis, Morris Plains, NJ),

final concentrations 7.5 μM and 21.9 μM; soluble calf skin collagen (Worthington Biochemical Corp., Freehold, NJ), final concentration 258 μg/ml; ristocetin (BioData, Horsham, PA), final concentrations 0.87 mg/ml and 1.30 mg/ml; arachidonic acid (Sigma, St. Louis, MO), final concentration 543 μg/ml; and human α-thrombin (a gift of John Fenton, Albany, NY), final concentration 0.2 U/ml.

Platelet Factor 4 and β-Thromboglobulin Assays

Platelet factor 4 (PF_4) and β-thromboglobulin (βTG) levels were measured in platelet lysates using the Asserachrom® PF_4 and βTG enzyme immunoassays (Diagnostica Stago, Asnieres-sur-Seine, France).

Statistical Methods

Probabilities were calculated using the resampling method utilizing at least 10,000 iterations [19].

RESULTS

Platelet vWF

Decreased platelet vWF ristocetin cofactor activity levels were observed in 10 of the 30 patients tested (ranging from 0.29 to 0.43 U/10^9 platelets, normal range 0.44 to 1.15 U/10^9 platelets), and five additional patients had borderline low values (0.45 U/10^9 platelets) (Table I). There was a significant difference between the values of the patients and the normal controls (normals: n = 18, mean = 0.81, SD = 0.19; HPS patients: n = 30, mean = 0.49, SD = 0.14, $P < 0.0001$) (Fig. 1A). Because the normal control group was comprised of adults, we also compared just the adult patients (n = 21) with the control group and found that the difference remained significant ($P < 0.0001$). The values for the younger patients were even more decreased compared with the adult patients and were significantly different from the control group. The platelet vWF antigen level was normal in all patients tested (normal range, 0.09–0.57 U/10^9 platelets), and there was not a statistically significant difference in the adult patients compared with the normals (normals: n = 18, mean = 0.33, SD = 0.12; adult HPS patients: n = 21, mean = 0.27, SD = 0.13, $P > 0.05$). The multimeric structure of the platelet vWF appeared normal in all 30 patients tested.

Plasma vWF and Factor VIII

Of the 44 patients tested, only one had low plasma vWF activity (31%) (this patient appeared to have concurrent von Willebrand's disease) and another had borderline plasma vWF activity (49%, normal range 48%–144%) (Table I). Although the other patients' values were within the normal range, there was a significant difference between the patients and the normals as a group at the level of $P \sim 0.03$ (normals: n = 25, mean = 93.1, SD = 18.9; HPS patients: n = 44, mean = 82.8, SD = 18.2) (Fig. 1B). The *P*-value was similar when only adult patients were compared with the control group. Four patients had low plasma vWF antigen levels (ranging from 32%–47%, normal range 50%–143%), although there was not a significant difference between the patients and the normals (normals: n = 25, mean = 93.2, SD = 24.2; HPS patients: n = 44, mean = 82.4, SD = 29.1, $P \sim 0.13$). Two patients had factor VIII levels below the normal range (45% and 46%, normal 50%–150%). Both of these patients also had low platelet vWF activity levels, and one of them had a borderline plasma vWF antigen level (50%). The multimeric structure of the plasma vWF was normal in the majority of patients; however, there was a decrease in the highest molecular weight multimers accompanied by an increase in the low molecular weight multimers in 12 patients (Fig. 2). The area under the curve defined by the upper 25% of the gel (highest molecular weight multimers as demarcated by the upper 25% of pooled normal plasma) was 16.2% ± 4.8% (one SD) for this subset of patients; the area for 18 normal subjects was 23.8% ± 5.3%.

Platelet Aggregation and Release Reaction

Platelet aggregation and release reactions were performed on 30 HPS patients. Each patient exhibited markedly decreased or absent release of ATP to every agonist used. Platelet aggregation was observed with collagen, thrombin, and arachidonic acid in all patients tested. The aggregation responses elicited with ADP or epinephrine varied widely from no discernable aggregation to two waves with little or no ATP release. All patients displayed at least one wave of aggregation with 1.30 mg/ml of ristocetin.

PF4 and βTG Levels

PF4 and βTG levels measured in platelet lysates from 12 HPS patients, including four patients with low platelet vWF ristocetin cofactor activity but normal vWF antigen levels, were all within the normal range (PF_4, 4.1–27.9 μg/10^9 platelets; βTG, 18.54–45.82 μg/10^9 platelets).

Clinical Symptoms

Clinical bleeding symptoms are shown in Table I. We were unable to demonstrate a correlation between laboratory assays, bleeding times, and clinical bleeding symptoms.

DISCUSSION

We examined the platelet vWF activity and antigen levels in 30 patients with HPS and plasma vWF activity and antigen levels in 44 patients with HPS and attempted to correlate these values with bleeding times and patient histories. Although decreased levels of platelet vWF ac-

TABLE I. Clinical and Laboratory Data in HPS Patients*

Patient (age)	Plasma vWF activity	Plasma vWF antigen	Platelet vWF activity	Platelet vWF antigen	Factor VIII	Bleeding time	Clinical bleeding[a]	Decreased HMW multimers
1 (10)	63%	67%	48%	33%	87%	>20	Major	Yes
2 (40)	72%	62%	43%	15%	46%	N.D.	Minor	Yes
3 (54)	77%	57%	43%	20%	67%	N.D.	Bruising	Yes
4 (16)	107%	86%	N.D.	N.D.	107%	N.D.	Minor	
5 (16)	98%	86%	N.D.	N.D.	98%	N.D.	Minor	
6 (12)	88%	57%	49%	26%	65%	N.D.		
7 (10)	115%	168%	41%	22%	136%	N.D.	Bruising	
8 (8)	115%	108%	51%	14%	116%	N.D.	Major	
9 (4)	118%	133%	N.D.	N.D.	118%	>15	Bruising	
10 (9)	98%	123%	N.D.	N.D.	98%	N.D.		
11 (7)	84%	110%	N.D.	N.D.	84%	N.D.	Bruising	
12 (17)	77%	118%	45%	23%	124%	12.5	Minor	
13 (22)	67%	107%	33%	25%	119%	11.5	Minor	
14 (6)	68%	47%	45%	13%	75%	N.D.	Minor	Yes
15 (16)	68%	36%	45%	17%	53%	>20	Minor	Yes
16 (30)	70%	47%	45%	28%	65%	>20	Minor	Yes
17 (24)	49%	56%	62%	26%	55%	>20	Minor	Yes
18 (4)	69%	61%	29%	N.D.	N.D.	>20	Bruising	
19 (16)	61%	73%	48%	24%	88%	>15	Bruising	
20 (13)	60%	58%	51%	15%	81%	>20	Minor	
21 (5)	105%	75%	43%	16%	83%	N.D.	Bruising	
22 (5)	89%	130%	68%	20%	87%	N.D.	Bruising	
23 (7)	74%	63%	52%	13%	76%	N.D.		
24 (10)	88%	79%	65%	22%	117%	N.D.	Bruising	
25 (48)	82%	100%	N.D.	N.D.	N.D.	N.D.	Major	Yes
26 (31)	31%	32%	N.D.	N.D.	N.D.	N.D.	Major	
27 (32)	77%	104%	55%	37%	72%	N.D.	Minor	Yes
28 (38)	75%	50%	34%	25%	85%	N.D.	Minor	Yes
29 (35)	91%	100%	41%	23%	83%	N.D.	Minor	
30 (26)	89%	82%	33%	25%	105%	N.D.	Bruising	Yes
31 (40)	107%	70%	57%	28%	106%	N.D.	Minor	
32 (28)	98%	128%	48%	22%	143%	N.D.	Minor	
33 (21)	78%	72%	45%	26%	55%	N.D.	Minor	
34 (4)	73%	76%	N.D.	N.D.	N.D.	N.D.	Bruising	Yes
35 (7)	83%	55%	N.D.	N.D.	83%	N.D.	Bruising	
36 (38)	82%	94%	N.D.	N.D.	82%	N.D.	Minor	
37 (38)	94%	84%	N.D.	N.D.	94%	N.D.	Minor	
38 (14)	107%	100%	N.D.	N.D.	N.D.	N.D.		
39 (21)	99%	67%	N.D.	N.D.	77%	>15	Bruising	
40 (6)	61%	50%	41%	17%	45%	>15	Minor	
41 (33)	82%	94%	102%	45%	88%	7	Bruising	
42 (25)	95%	100%	46%	36%	115%	8	Minor	
43 (20)	80%	68%	65%	36%	94%	5	Minor	
44 (37)	81%	92%	N.D.	N.D.	N.D.	N.D.	Minor	
Normal range	48–144%	50–143%	44–115%	9–57%	50–150%	<8.5 min		

*HPS, high molecular weight; vWF, von Willebrand factor; N.D., not done.
[a]Bruising, bruising alone; minor, bruising, epistaxis, and other bleeding not requiring transfusion; major, bleeding requiring transfusion of red cells.

tivity were found in one third of the patients tested, no relationship with clinical bleeding was observed. The number of patients was not large, and a number of the patients we studied were children who did not have bleeding times performed and required limited blood draws; several also had not encountered a significant hemostatic challenge.

The number of α-granules present and the amount of the α-granule protein βTG have been reported as normal in the HPS patients studied [2,8]. We also found antigenic βTG, as well as antigenic PF_4 and vWF, other α-granule proteins, to be within normal limits. However, we observed that platelet vWF activity was significantly lower in the HPS patients ($P < 0.0001$) when compared to the normals studied. Thirty-seven percent of the HPS patients had abnormally low levels of platelet vWF activity and an additional 19% had borderline values.

We reported previously that platelet vWF activity and antigen levels in type I von Willebrand disease correlate better with the bleeding time than do the plasma vWF

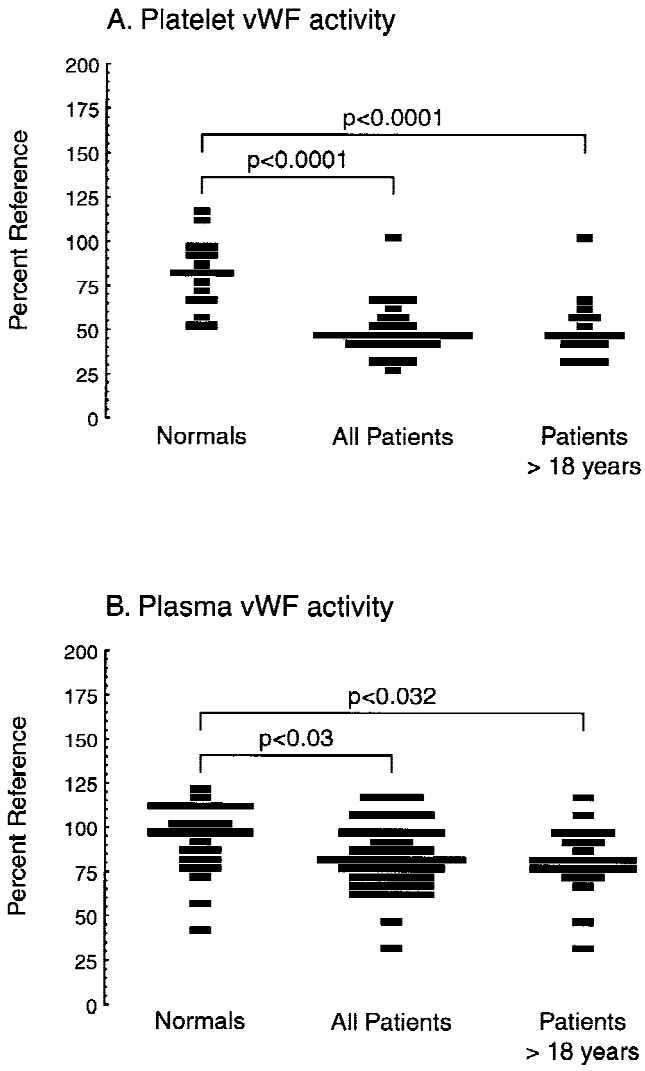

Fig. 1. Distributions of platelet and plasma vWF activity in normals and HPS patients. The number of patients or normal subjects is indicated by the length of each bar.

activity or antigen levels, which suggests to us that platelet vWF may be a more reliable predictor of clinical bleeding than plasma vWF [20]. Nonetheless, we were unable to discern a correlation of platelet vWF levels and bleeding symptoms in the HPS patients. The values of plasma vWF activity and antigen, which showed only borderline–low or no differences from the normal subjects, were also not predictive of bleeding symptoms in the HPS patients. The abnormalities of the multimeric structure in the plasma vWF of 12 of the HPS patients (Fig. 2) is under further study. Of these 12 patients, two have experienced major bleeding, seven minor bleeding, and three only bruising.

We found abnormalities of ATP release in the 30 HPS patients studied, consistent with the storage pool deficiency in these patients, whereas abnormalities in platelet aggregation were variable. Although it has been reported

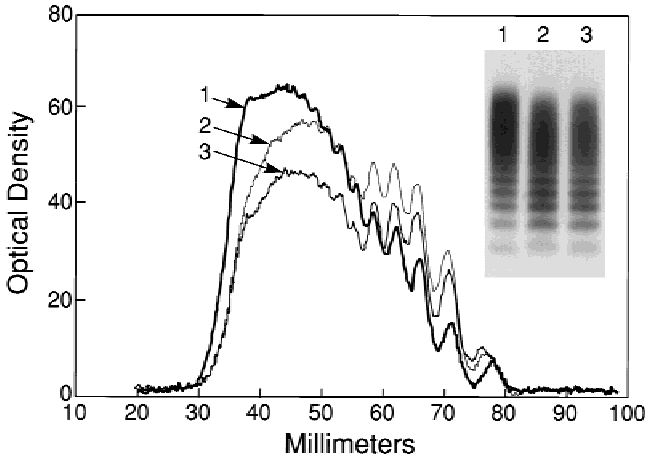

Fig. 2. Densitometric scans and autoradiographs of plasma vWF multimers in two representative patients who show a decrease in high molecular weight multimers and an increase in low molecular weight multimers. Autoradiograph: normal pooled plasma is shown in lane 1 and two patients are shown in lanes 2 and 3; densitometric tracing: normal pooled plasma is shown as the heavy tracing and the two patients are shown as the thinner tracings. Equal quantities of vWf antigen were loaded on the gel.

that aggregation of platelets by collagen is impaired in HPS patients, we observed collagen-induced aggregation using a relatively high dose of bovine collagen in all of the HPS patients we analyzed.

vWF levels and platelet function make additive contributions to hemostatic integrity. The severity of bleeding in Puerto Rican patients with HPS is reported to be increased when they also have low-normal or low levels of vWF antigen [14]. Similarly, swine with both plasma vWF deficiency and platelet storage pool deficiency have a more severe bleeding tendency than pigs with von Willebrand disease alone [13,14]. Therefore, it is possible that the lower levels of plasma and platelet vWF observed in our series of HPS patients may contribute to their bleeding tendency; however, long-term follow-up of these patients and testing of additional HPS patients will be necessary to test this hypothesis.

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